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Manipulations capable of repealing host tolerance to induce T cell-mediated prostate tissue specific responses are of central importance to immunotherapeutic approaches to prostate cancer treatment. Hence in the current proposal, we test whether androgen ablation (by castration) can induce T cell responses targeting murine prostate epithelial and tumor cells. We previously showed that castration of TRAMP mice results in prostate and tumor infiltration by antigen presenting cells (APC's) as well as CD4+ and CD8+ T cells. These studies completed our original **Specific Aim 1** and suggested that castration can prime host responses amenable to immunotherapeutic potentiation. Over the last year we have essentially addressed **Specific Aims 2 and 3** by showing that androgen ablation can facilitate/potentiate the effectiveness of novel immunotherapies (anti-CD40 and anti-CTLA-4 treatment) to enhance T cell-mediated TRAMP tumor inflammation for prostate cancer treatment. Hence, we have extended our studies by conducting a number of exploratory experiments to elucidate central mechanisms whereby androgen ablation augments T cell-mediated immunity.

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INTRODUCTION

Our previous studies pertaining to T cell costimulatory-based immunotherapies of murine prostate cancer [1-9], as well as antitumoral immunotherapeutic studies by our collaborators and others [10-14], underscore two principle points: 1) immunotherapies (including CTLA-4 blockade) may prove particularly effective when tumor-induced impairments in host immune function are minimized -- for instance, by diminishing tumor burden and/or reducing the capability of tumor cells to elaborate immune inhibitors; and 2) prior interventions that successfully overcome host tolerance to prime a prostate-specific, cell-mediated immune response should greatly facilitate antitumoral responses bolstered by immunotherapies such as CTLA-4 blockade. Hence, to address these two points within the context of developing potent immunotherapeutic approaches for the treatment of prostate cancer, we originally proposed to test whether androgen ablation might prove useful as a "first step" in converting prostate tumors into their own "*in situ vaccines*" to facilitate a response to immunotherapy. The mechanism(s) we envisioned whereby androgen ablation might induce/facilitate immunotherapeutic responses are encompassed in two non-mutually exclusive paradigms. In the first paradigm, androgen ablation reduces tumor burden thus reducing the number of tumor cells that must be eliminated by an immune response raised by CTLA-4 blockade. Additionally, reduction of tumor burden or downregulation of tumor cell function by androgen ablation may partially alleviate some immuno-suppressive effects of the tumor upon APC and T cell function. In the second paradigm, androgen ablation results in the recruitment of immune cells into tumor sites. Moreover, androgen ablation-induced prostate cell death might result in greater availability tumor-derived antigens that can be appropriate by host APC for presentation to induce antigen-specific activation of T cells whose action can be potentiated by immunotherapies such as CTLA-4 blockade -- somewhat analogous to the capability of CTLA-4 blockade to markedly exacerbate pre-existent pathologic inflammatory processes such as murine experimental allergic encephalomyelitis.

BODY

Our current DOD proposal is intended to address our overall hypothesis that: **Androgen ablation combined with CTLA-4 blockade immunotherapy can diminish/prevent the progression of prostate cancer that inevitably occurs following androgen ablative therapy alone.** The following annual report summarizes our progress between January to December 2001 in testing this overall hypothesis by addressing three separate aims we previously outlined:

Specific Aim 1. To Histologically and functionally characterize immune cells that infiltrate prostate tumors following androgen ablation.

Specific Aim 2. To test the hypothesis that *in vivo* CTLA-4 blockade can diminish the progression of prostate cancer that inevitably occurs following androgen ablative therapy alone.

Specific Aim 3. To test the hypothesis that *in vivo* activation of host antigen presenting cells can potentiate antitumoral responses raised by the combination of androgen ablation and CTLA-4 blockade immunotherapy.

To date, we have completed the objectives of our original *Specific Aim 1*. Data from these experiments showing that castration raises T cell-mediated inflammation within the murine prostate were comprehensively summarized in our previous Technical Report to the DOD for the interval between January and December 2000. Over the last year, we have been conducting experiments, as outlined in our **Statement of Work**, to complete

experiments outline in our original *Specific Aims 2 and 3*. These experiments have required hybridoma production of 22 mg of anti-CTLA-4 (9H10 cells) monoclonal antibody, the breeding homozygote TRAMP mice with C57BL/6 mates to generate 150 heterozygote males for establishment of treatment cohorts, and hybridoma production of 210 mg of AntiCD40 rat monoclonal antibody (10C8 cells).

Over this past year, we have been able to demonstrate that castration-induced T cell-mediated inflammation in the prostates of TRAMP mice is apparently potentiated by systemic (intraperitoneal) combined anti-CD40 and anti-CTLA-4 administration. In these experiments, cohorts of twelve week-old adult post-pubertal TRAMP mice (n=25 each cohort) underwent castration on Day 0 of the experiment. Non-castrated cohorts of mice served as non-androgen ablated controls for this study. At day 3 following castration, the treatment cohorts received a single IP injection of anti-CD40 antibody (500 ug) to promote host APC activation -- including especially those APC's infiltrating prostate tissues following castration. Control (non-immunotherapy-treated) cohorts received irrelevant control IgG. Furthermore, mice that received anti-CD40 additionally received I.P. injections of 100 ug of anti-CTLA-4 on days 4, 7 and 10 following castration. Likewise, non-immunotherapy-treated mice received corresponding control IgG. All mice were sacrificed two weeks following completion of antibody treatment. Immunohistochemical analysis of prostates recovered from non-castrated TRAMP mice (Figures 1 A&B) revealed that combined anti-CD40 + anti-CTLA-4 treatment (Figure 1B) failed to significantly increase levels of prostate CD3 T cells within the prostate.

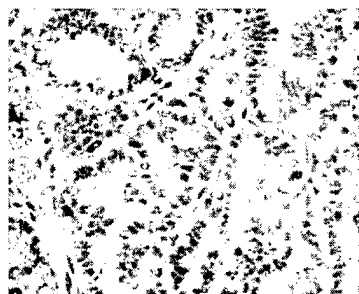


Figure 1A



Figure 1B

In contrast, and as we have previously shown, non-immunotherapy-treated castrated TRAMP mice revealed increased levels of prostate CD3 T cells (Figure 2A). Finally, administration of combined anti-CD40 + anti-CTLA-4 treatment to castrated TRAMP mice appears to further enhance levels of CD3 T cells infiltrating the TRAMP prostate (Figure 2B).

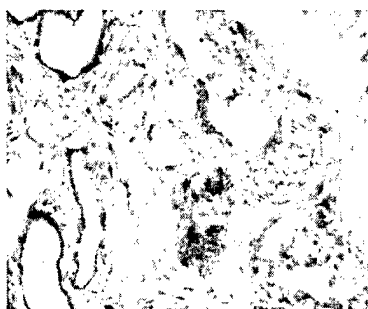


Figure 2A

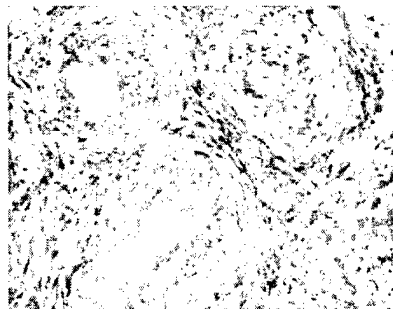


Figure 2B

In summary, our aforementioned studies conducted over the last years strongly suggest that androgen withdrawal (by castration) is capable of inducing a form of T cell-mediated prostate inflammation in TRAMP mice that is amenable to potentiation by anti-CD40/anti-CTLA-4 immunotherapy. Formal quantification of this T cell response targeting TRAMP tumors following castration + anti-CTLA-4/anti-CD40 immunotherapy is currently underway and will be completed over this next year.

At present, we have additionally initiated a number of exploratory experiments to extend our studies to elucidate mechanisms whereby castration might induce prostate inflammation to facilitate immunotherapeutic responses within the prostate. These extended studies are of particular interest to us since we have recently shown (and published [15]) that androgen ablative therapy in men with prostate cancer raises T cell-mediated prostate inflammation similar to that observed in the TRAMP model (as well as in normal C57BL6 mice). Our current line of investigation is to test the general hypothesis that androgen withdrawal augments host T cell-mediated immunity by lowering the overall threshold for T cell co-stimulatory activation/proliferation. The impetus for this avenue of study has been provided by previously published reports that: 1) castration of post-pubertal animals can potentiate B and/or T cell-mediated responses against various pathogens or tissue allografts [16]; 2) castration profoundly stimulates T and B cell lymphopoiesis in postpubertal rodents [16, 17] -- implying that certain restrictions on lymphocyte proliferation and/or activation might be lifted upon withdrawal of androgen and; 3) clinical prostate cancer progression might be modulated by systemic host immune responses apparently triggered upon androgen deprivation [18-21].

To date, we have been able to demonstrate that T cell and B cell (IgG) responses against the nominal antigen, OVA, are enhanced by castration (androgen withdrawal) in vaccinated C57BL/6 mice (Figure 3). Similarly, T cells recovered from castrated mice appear to exhibit increased reactivity in one-way allo-MLR assays (Figure 4). We have further observed that castration apparently triggers *polyclonal* T cell expansion *in vivo* as evidenced by: i) a broad and uniform increase in CD4+ and CD8+ T cell numbers within thymii, lymph nodes and spleens of castrated mice and ii) the absence of associated TCR VB clonotype distributional skewing accompanying this T cell response to castration (*data not shown*). To further establish a mechanistic basis for enhanced T cell "responsivity" that occurs following castration, in recent studies we have observed that T cells recovered from castrated mice proliferate at higher rates in response to anti-CD3/anti-CD28-mediated costimulation compared to costimulated T cells recovered from sham-castrated mice (Figure 5). Collectively, the above studies suggest that castration lowers the overall threshold that is required for T cell costimulatory proliferation/activation. Such studies provide compelling support for the potential of androgen withdrawal as a viable means to facilitate/potentiate responses to immunotherapy. Hence, further experiments are currently underway to comprehensively complete work proposed in our original *Specific Aims 2 & 3*, and to further provide preliminary data that might support future grant submissions intended to explore the effect of hormone manipulation on T and B cell-mediated antitumoral and auto-immunity.

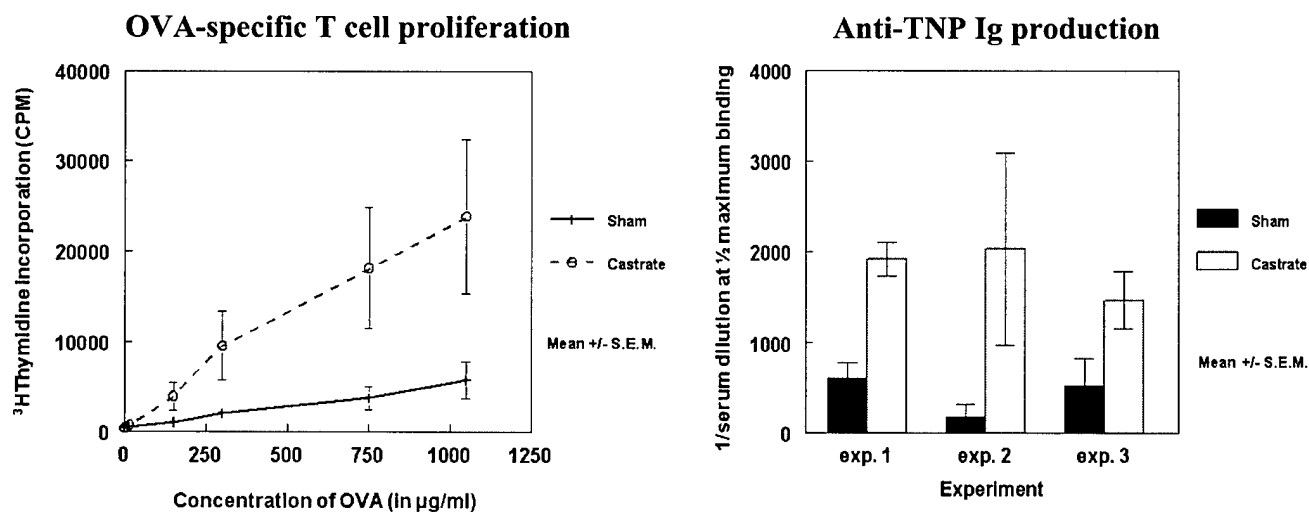


Figure 3: **OVA-specific T cell proliferation:** C57BL/6 mice (7-10 wks of age) were either castrated or sham operated. Two (2 experiments) or 6 (3 experiments) wks later, animals were immunized with 50µg TNP₂₅OVA in CFA or PBS in CFA (data not shown). After 14 days, mice were sacrificed. T cell-enriched splenocytes were stimulated by irradiated adherent cells, also obtained from splenocytes, and increasing amounts of OVA or medium in the presence of dihydrotestosterone at a physiological level (cultures from castrated mice) or equal amounts of ethanol (cultures from sham treated animals). Cultures were incubated for 4 days, cells were pulsed with 1µCi ³HThymidine/well and harvested after an o/n incubation at 37 °C. Graph is representative for five independent experiments with 3 mice per treatment group each. **Anti-TNP Ig production:** Anti-TNP production was measured in the serum from mice used in the experiments described above using ELISA-technique. The experiments demonstrated in the graph above are representative of in total five experiments whereby significant differences in the anti-TNP Ig production between sham-treated and castrated animals was observed in the three above demonstrated experiments.

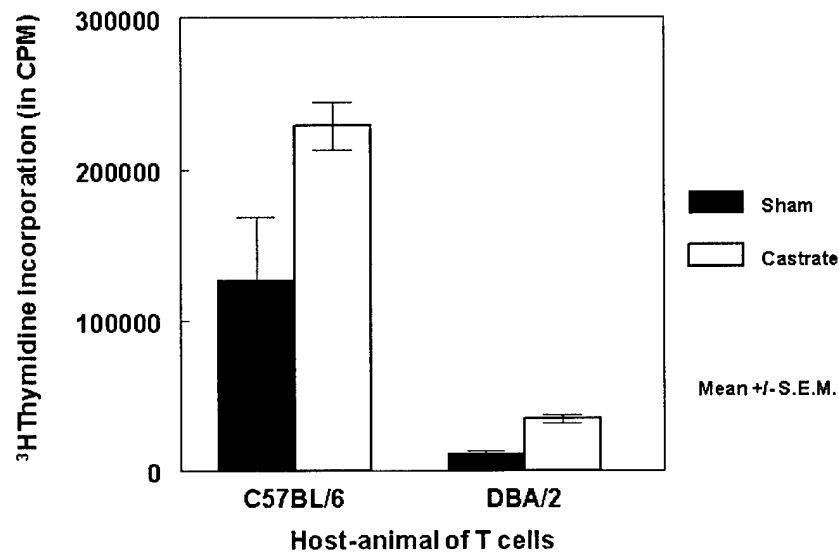


Figure 4: Proliferation of T cells stimulated in an allo-MLR assays. C57BL/6 mice (8 wks of age) were either castrated or sham operated. 7 days later, surgically treated C57BL/6 mice and non-treated DBA/2 mice were sacrificed. A portion of splenocytes was irradiated with 25Gy and used as stimulators. The remaining splenocytes were enriched for T cells (purity >97%, evaluated by flowcytometry) and used as responders. 0.35×10^6 T cells from C57BL/6 mice were incubated together with 0.5×10^6 stimulator cells from DBA/2 in the presence of dihydrotestosterone at a physiological level (cultures from castrated mice) or equal amounts of ethanol (cultures from sham treated animals). Additional cultures contained T cell from DBA/2 mice and stimulator cells from C57BL/6 mice using similar conditions. Cultures were incubated for 4 days, pulsed with $1\mu\text{Ci}$ $^3\text{HThymidine}$ /well and harvested after an o/n incubation at 37°C . Graph is representative for two independent experiments.

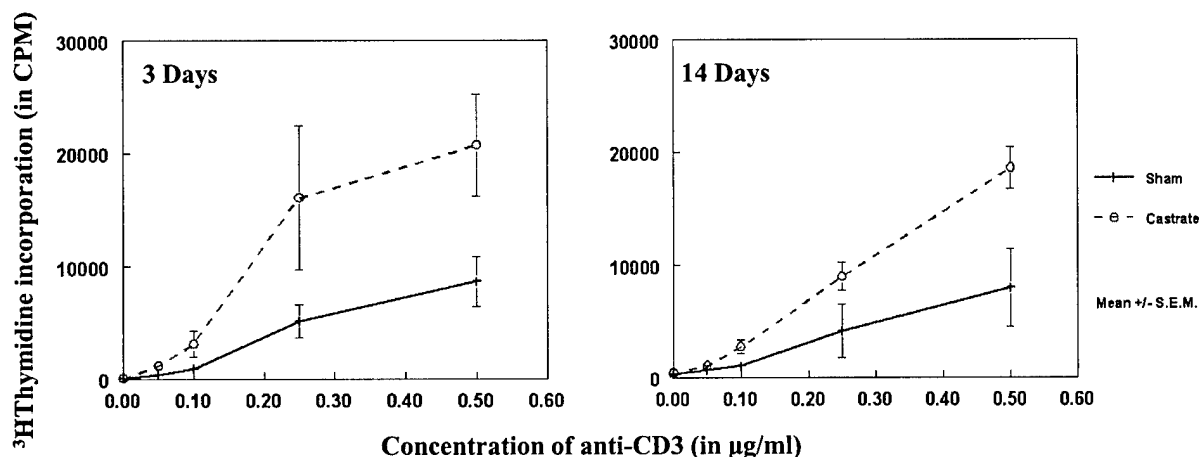


Figure 5: T cell proliferation after costimulation with anti-CD3 and anti-CD28. C57BL/6 mice (8-14 wks of age) were either castrated or sham operated. Three or 14 days later, mice were sacrificed and splenocytes were enriched for T cells (purity >97%, evaluated by flow cytometry). Plates were coated with increasing amounts of anti-CD3 (100µl/well) or PBS (data not shown), incubated at 37°C for 1.5 h and washed three times with PBS. 0.2×10^6 T cells were added to each well in 200µl medium supplemented with anti-CD28 (1µg/ml) and dihydrotestosterone at a physiological level (cultures from castrated mice) or ethanol (control cultures). After 48 h incubation, cells were pulsed with 1µCi $^3\text{HThymidine}$ /well and harvested after an o/n incubation at 37 °C. Graph is representative of three independent experiments at each time point with 3 mice per treatment group.

KEY RESEARCH ACCOMPLISHMENTS

- Completed Specific Aim 1. Established that castration in TRAMP mice induces prostate tumor infiltration by mononuclear inflammatory cells.
- Nearly completed Specific Aims 2 & 3.
- Initiated exploratory studies to examine the effects of androgen withdrawal on T and B cell regulation/function.

REPORTABLE OUTCOMES TO DATE

Kwon ED, Foster BA, Hurwitz AA, Madias C, Allison JP, Greenberg NM, Burg MB. Elimination of Residual Metastatic Prostate Cancer Following Surgery and Adjunctive CTLA-4 Blockade Immunotherapy. *Proc. Natl. Acad. Sci.*, 96(26):15074-9, 1999.

Hurwitz AA, Foster BA, **Kwon ED**, Truong T, Choi EM, Greenberg NM, Burg MB, Allison JP. Immunotherapy of primary prostate cancer in a transgenic model using a combination of CTLA-4 blockade and tumor cell vaccine. *Cancer Research*, 60(9):2444-2448, 2000.

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CONCLUSIONS

Observations emanating from our current studies collectively support the hypothesis that castration causes prostate tumor infiltration by both antigen presenting cells and T cells with potential antitumoral effector capabilities. Such a response might serve as a useful “*first step*” to prompt host prostate tumors (both primary or metastatic) to act as their own *in situ* vaccines to “*jump-start*” anti-prostate cancer immune responses. To date, we have characterized this response and have shown that two promising immunotherapies, CTLA-4 blockade and/or anti-CD40 treatment, can act synergistically with castration to induce T cell-mediated responses against prostate tumors in the immunocompetent TRAMP mouse. Our work has further provided the basis for clinical phase I testing of CTLA-4 blockade for the treatment of prostate cancer. Additionally, our current studies have provided the scientific foundation for a DOD-sponsored clinical phase II trial that will begin later this year to test whether androgen ablative therapy + anti-CTLA-4 administration can be used to induce/potentiate T cell-mediated anti-prostate tumor responses in men with advanced prostate cancer. Finally, our current exploratory studies may elucidate additional mechanisms whereby hormone therapy modulates T cell-mediated immunity. Such studies may reveal additional novel strategies to markedly potentiate antitumor responses during immunotherapeutic intervention during prostate cancer treatment.

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APPENDICES: NONE